PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Robert G. Korneluk et al.	Art Unit:	1635
Serial No.:	09/974,952	Examiner:	Janet L. Epps
Filed:	October 9, 2001	Customer No.:	21559
Title:	DETECTION AND MODULATION OF IAPS AND NAIP FOR THE DIAGNOSIS AND TREATMENT OF PROLIFERATIVE DISEASE		

Commissioner For Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132 OF ROBERT G. KORNELUK, PH.D.

1. I am an inventor on the above-captioned patent application.

2. I have read the Office Action mailed November 20, 2002.

3. We have successfully reduced to practice the present invention using techniques known to those skilled in the art of antisense oligonucleotide technology at the time the priority application for the presently claimed invention was filed. Specifically, we showed the effect of XIAP down-regulation by antisense oligonucleotides on human non-small cell lung cancer (NIH-H460) growth *in vivo*. In animal models, we demonstrated that the G4 antisense oligonucleotide at 15 mg/kg had significant sequence-specific

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growth inhibitory effects on human H460 tumors in xenograft models of SCID/RAG2-immunodeficient mice by systemic intraperitoneal administration. Systemic antisense oligonucleotide administration was associated with an 85% down-regulation of XIAP protein in tumor xenografts. The combination of 15 mg/kg G4 AS ODN with 5 mg/kg vinorelbine significantly inhibited tumor growth, more than either agent alone. These studies indicated that down-regulation of XIAP is a potent death signal in lung carcinoma cells and is able to inhibit tumor growth *in vivo*. These studies support the contention that the *in vivo* administration of antisense oligonucleotides complementary to XIAP SEQ ID NO:3 was enabled as a cancer therapy at the time applicants' priority document was filed.

4. The antisense oligonucleotides were selected using methods outlined in our disclosure. Specifically, we selected ninety-six oligonucleotide sequences complementary to a portion of XIAP (each sequence having nineteen nucleotides) (SEQ ID NOs: 1 through 96; Table 1), from a region approximately 1 kb upstream of the start codon to approximately 1 kb downstream of the stop codon of the XIAP cDNA sequence.

5. As described in our specification, the XIAP synthetic library of 96 antisense oligonucleotides was first screened *in vitro* to identify antisense oligonucleotides that decreased XIAP protein levels. Specifically, T24 cells (1.5×10^4 cells/well) were seeded in wells of a 96-well plate on day 1, and were cultured in antibiotic-free McCoy's medium for 24 hours. On day 2, the cells were transfected with XIAP antisense oligonucleotides. On day 3, XIAP RNA levels were measured using quantitative real-time PCR techniques. At day 4, XIAP protein levels were measured by ELISA (Exhibit B-A, C, E, G, I, and K) and total cellular protein was measured biochemically (Exhibit B-B, D, F, H, J, and L); and used to normalize the XIAP protein levels). These results were

compared to a mock transfection sample (treated with the transfection agent without oligonucleotide DNA, and then processed as for the other samples).

These methods identified 16 antisense oligonucleotides that decreased XIAP protein levels relative to control cells that were mock transfected. As expected, the ability of an antisense oligonucleotide to decrease XIAP protein levels correlated with its ability to decrease XIAP mRNA levels (Exhibit C-A and B). Sixteen of these oligonucleotides decreased by at least 50% levels of XIAP protein or mRNA levels. The G4 AS ODNs exhibited the strongest down-regulating effect on XIAP protein, reducing XIAP protein levels by 62% within twenty-four hours after the end of transfection (Exhibit D-A and D-B). As shown in Exhibit E, cells treated with G4 AS ODNs underwent morphological changes characteristic of apoptosis, including chromatin condensation and nuclear DNA fragmentation. Few control cells showed these morphological changes. Forty-eight hours after transfection, G4 AS ODNs reduced H460 cell growth 55% relative to untreated controls (Exhibit F). *In vivo* studies with lung carcinoma and breast carcinoma cells were then carried out.

6. SCID-RAG2 mice were inoculated with H460 human lung carcinoma cells (subcutaneous shoulder injection of 10^6 cells) and treatments with G4 and F3 AS PS-ODNs, as well as a scrambled control, were initiated three days after tumor inoculation. ODN injections were administered intraperitoneally at 12.5 mg/kg three times a week for three weeks. At the end of the treatment period, mean tumor sizes in the groups treated with either G4 or F3 antisense oligonucleotides were ~ 50 % smaller relative to tumor size in a control group treated with a scrambled control oligonucleotide (Exhibit G).

7. The treatment protocol described above was also tested on female SCID-RAG2 mice inoculated orthotopically with MDA-MB-435/LCC6 human breast carcinoma

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cells. Two weeks after the last treatment (day 35) tumor volumes of mice treated with F3, C5 or G4 AS ODNs were 70 %, 60 % and 45 % smaller than vehicle controls (Exhibit H).

8. G4 AS ODNs in SCID-RAG2 mice bearing xenografts of H460 human non-small-cell lung tumors implanted subcutaneously. Saline-treated control tumors grew reproducibly to a size of 0.75 cm^3 within approximately 24 days (Exhibit I). ODN treatments were initiated three days after tumor cell inoculation. G4 AS ODNs (5 to 15 mg/kg) were administered using a treatment schedule of intraperitoneal injections given on days 3-7, 10-14, and 17-21 (once a day). The treatment with 5 or 15 mg/kg G4 AS ODNs greatly delayed tumor growth: on day 24 mean tumor sizes were 0.75, 0.45 and 0.29 cm^3 in control, 5 and 15 mg/kg treated groups, respectively (Exhibit I). There was a dose-dependent inhibition of tumor growth. Tumor size in mice treated with 15 mg/kg G4 AS ODNs was significantly smaller than in control groups, and represented 39% of control mean tumor size. In contrast, administration of G4 scrambled oligonucleotides at 15 mg/kg provided no therapeutic activity. None of the mice treated with ODNs displayed any signs of toxicities, and both doses of ODNs were well tolerated. A dose of 15 mg/kg was selected for the future combination treatment regimens with anticancer drugs.

9. To correlate G4 AS ODN tumor growth inhibitory effects with XIAP protein expression, we examined the changes in XIAP expression at the end of the *in vivo* treatment with 15 mg/kg of G4 AS and scrambled oligonucleotides. At day 21 or 24 post-tumor inoculation when tumors reached 1 cm^3 in size, tumors were harvested and lysates from tumor homogenates were used for western blot analysis. XIAP and β-actin antibodies against the human protein were used, allowing for determination of human

XIAP levels obtained from tumor cells specimens without contamination from XIAP derived from mouse cells. XIAP protein levels in tumors treated with G4 AS ODNS were significantly reduced to approximately 85% of control tumors (P <0.005) (Exhibit J). XIAP protein in tumors treated with G4 Scrambled oligonucleotides were reduced in size by 24% of control tumors. These results indicated that inhibition of H460 tumor growth by G4 AS ODNS correlated with the down-regulation of XIAP protein expression.

10. To evaluate whether XIAP AS ODN administration results in direct tumor cell kill, we examined the histology of tumors after treatment both for morphology and ubiquitin immunostaining (Exhibit K-A and B). At day 21 or 24 post-tumor inoculation, tumors treated with 15 mg/kg of G4 AS, scrambled oligonucleotides, or saline control were excised, sectioned, and stained with hematoxylin and eosin. The results demonstrated that tumors in animals administered given XIAP AS ODNs treatment contained an increased number of dead cells, identified morphologically by their amorphous shape and condensed nuclear material.

12. As described in our specification, we also combined treatments of G4 AS ODNs with known a chemotherapeutic agent used for lung cancer treatment. The therapeutic efficacy of vinorelbine in the presence and absence of G4 AS ODNs or scrambled oligonucleotides. Treatment was initiated on day 3 after tumor inoculation. Exhibit L presents the *in vivo* efficacy results for 5 mg/kg and 10 mg/kg doses of vinorelbine given to H460 tumor-bearing mice and compared with saline controls. Each of the two regimens induced significant tumor growth suppression in a dose-dependent manner without showing significant signs of undesirable toxicity (i.e., body weight loss). When administration of G4 AS ODNs (15 mg/kg) was combined with vinorelbine (5 mg/kg) for the treatment of H460 tumors, a more pronounced delay of H460 tumor

growth was observed compared to either treatment administrated alone (Exhibit L). Again, the mice did not show any significant signs of toxicity (i.e., body weight loss). Mean tumor size in mice treated with 5 mg/kg vinorelbine in the presence or absence of G4 AS or Scrambled oligonucleotides was compared on day 29 (Exhibit L A and B). The tumor sizes in the group receiving combination therapy was 0.22 ± 0.03 cm^3, significantly smaller than the tumor sizes of groups receiving any other treatment (tumor size in control mice receiving 5 mg/kg vinorelbine alone or a combination of vinorelbine and G4 scrambled oligonucleotides was 0.59 ± 0.04 and 0.48 ± 0.05 cm^3, respectively).

13. In sum, using routine methods described in our specification at the time of filing, we have now demonstrated the *in vivo* therapeutic efficacy of antisense oligonucleotides for enhancing apoptosis in a cell of a mammal and for the treatment of a patient diagnosed as having a proliferative disease.

14. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: May 5/03

Robert G. Korneluk, Ph.D.